enok encodes a *Drosophila* putative histone acetyltransferase required for mushroom body neuroblast proliferation

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Mushroom bodies in the *Drosophila* brain are centers for olfactory learning and memory. We have previously shown that the mushroom bodies comprise three types of neurons with distinct axonal projections. These three types of neurons are generated sequentially from common neuroblasts [1]. We report here the identification of a gene that we have named *enoki mushroom (enok)*, which when it is mutated gives rise to mushroom bodies with reduced axonal structures. *enok* encodes a putative histone acetyltransferase (HAT) of the MYST family, members of which have been implicated as important modulators of transcriptional activity [2]. A single amino acid change in the zinc finger motif of the putative catalytic HAT domain gives the same phenotype as a null allele, and this finding indicates the importance of HAT activity to Enok's function. Further phenotypic analysis demonstrates that the mushroom body defect is due to an arrest in neuroblast proliferation rather than a failure of either cell fate switching or axon branching. Clonal analyses in the wing discs and the ovaries suggest that *enok* is essential for normal cell proliferation in some, but not all, tissues. Our results provide in vivo evidence for essential functions of a histone acetyltransferase in the construction of the *Drosophila* brain.

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Results and discussion

Molecular and genetic characterization of the *enok* gene

The *enok* gene was originally identified because its alleles failed to complement a small deficiency, *Df(2)gek*^{D23} (*D23*), at cytological location 60B. This deficiency also includes an essential part of the *genghis khan (gek)* gene [3]. Using an ethyl methane sulfonate (EMS) mutagenesis screen for the failure to complement *D23* lethality, we defined a complementation group containing 12 alleles. When these alleles proved not to be within *gek*, we sequenced genomic DNA adjacent to *gek* and identified the *enok* open reading frame, in which two of the 12 alleles were eventually mapped molecularly (see below).

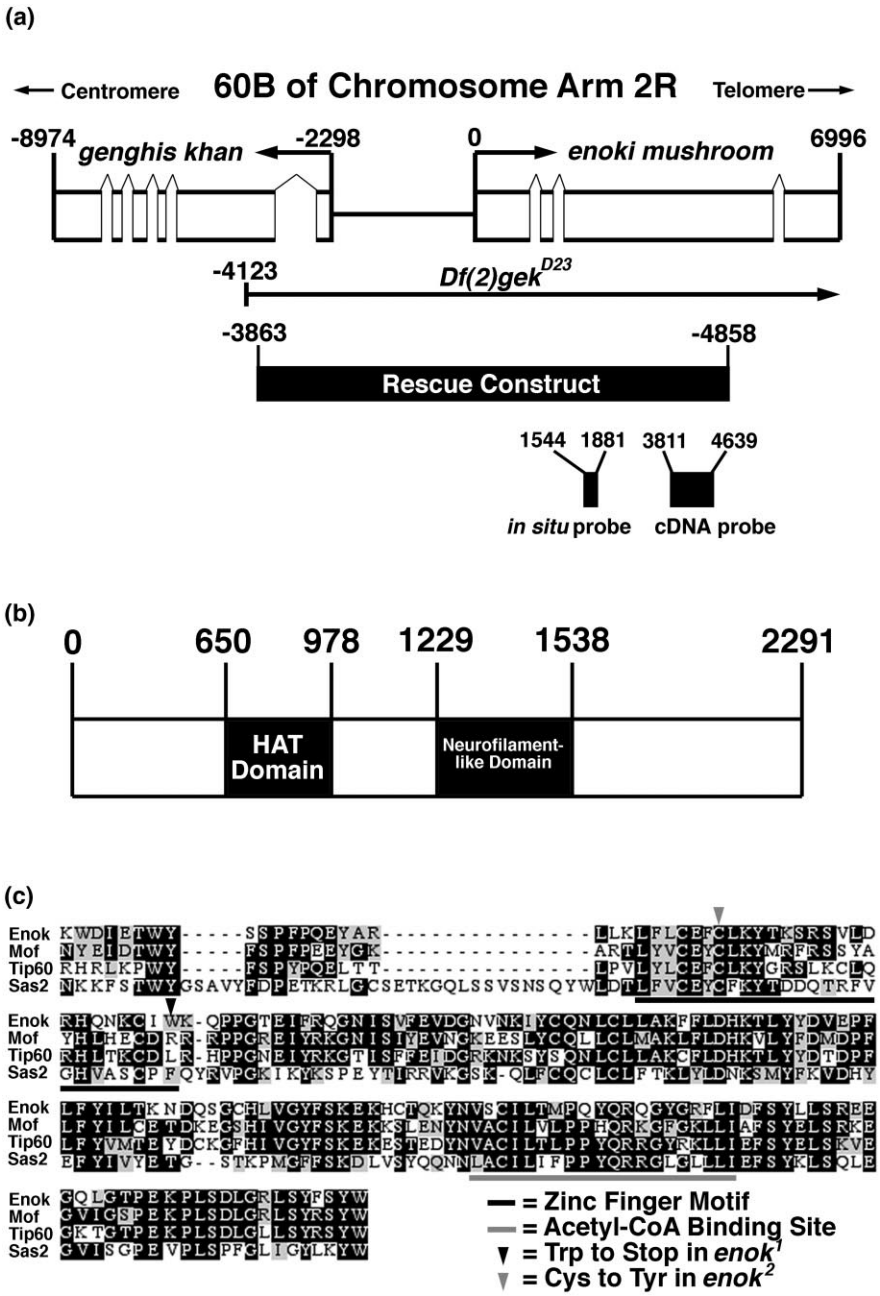
The *enok* cDNA contains an open reading frame predicted to encode a protein of 2291 amino acids (Figure 1a). The region of highest similarity to other proteins is the putative histone acetyltransferase (HAT) domain (Figure 1b). HAT proteins have recently been recognized as important modulators of transcriptional activity. Within the HAT domain, the predicted Enok protein is highly similar to other members of the MYST family of histone acetyltransferases, including *S. cerevisiae* SAS2 [4], *Drosophila* Mof [2], and human TIP-60 [5]. Enok's predicted catalytic domain is most similar to that of Mof, a *Drosophila* HAT protein required for dosage compensation [2]. Within the conserved histone acetyltransferase region, MYST family acetyltransferases share two motifs of particular interest, both of which are present in Enok (Figure 1c). The first of these is a predicted C_2HC zinc finger motif, a possible site involved in protein-protein interactions or DNA binding [2] and the second motif of interest has been shown in similar proteins to bind acetyl-CoA [6].

In addition to the HAT domain, Enok contains a region that shows similarity to the neurofilament H proteins in mammals. The closest relative is the rabbit neurofilament H protein, which shares 28% identity and 43% similarity with Enok over a 319 amino acid stretch. The region of similarity to Enok is in a part of the neurofilament that has been shown to serve as a target for serine/threonine kinases [7]. However, the numerous Lys-Ser-Pro (KSP in the single-letter code) repeats, which serve as targets for phosphorylation in the neurofilament proteins [8], are largely absent from Enok.

In situ hybridizations revealed ubiquitous expression of the *enok* transcript throughout embryonic life (data not shown). The transcript is particularly abundant in early embryos, and this finding indicates a maternal contribution.

Among the 12 EMS alleles, we have identified two as being point mutations within the conserved putative HAT domain (Figure 1c). The first allele, *enok*^{1}, is a nonsense mutation in the HAT domain that is predicted to truncate

Figure 1



The *enok* gene and protein. **(a)** The genomic position of *enok* in relation to *gek* is shown. The beginning and end points of the two genes, the *D23* deficiency, the *enok* rescue construct, and the in situ probe are shown in base pairs, with 0 being the start codon of *enok*. The rescue construct contains only part of the *enok* reading frame because it originated from a construct extending farther on the centromeric end that was previously used to rescue $Df(2)gek^{D23}$ [3]. A full-length cDNA was isolated from the LD 0–24 hr *Drosophila* phagemid cDNA library. **(b)** The protein predicted from the *enok* cDNA is shown. Positions of the domains of interest are shown according to their amino acid numbers. **(c)** An alignment of Enok's putative HAT domain with those from other MYST family members is provided. Amino acid identities are boxed in black, and conservative changes are indicated by gray shading. Also indicated are the predicted zinc finger domain, the putative acetyl-CoA binding site, the position of the predicted tryptophan-to-amber mutation of $enok^1$, and the position of a cysteine-to-tyrosine mutation in $enok^2$.

the protein at the end of the zinc finger motif (Figure 1c). The second allele, $enok^2$, contains a missense mutation that changes one of the zinc finger's conserved cysteines to a tyrosine (Figure 1c). This change likely leads to a disruption in the structure of the zinc finger. Since the *D23* deficiency perturbs more than one gene, we have made use of the $enok^1$ and $enok^2$ alleles in our subsequent studies.

Animals that are homozygous mutants for *enok* develop slowly and die before or during the pupal stage. Homozygotes tend to enter the wandering third instar larval stage at about 7 days after larval hatching (ALH) at 25°C, while their heterozygous counterparts take only 3.5–4.0 days. Approximately 50% of $enok^-$ animals die before puparium formation. Those that pupate do so at around 10 days ALH at 25°C as compared to 4.0–4.5 days for heterozygotes. Homozygotes never eclose as adults. In all of these respects, the phenotypes for homozygous *D23*, $enok^1$, and $enok^2$ individuals are indistinguishable.

enok is essential for the normal development of the mushroom bodies

Since the general lethality of $enok^-$ animals reveals little about the cellular functions of Enok, we undertook a

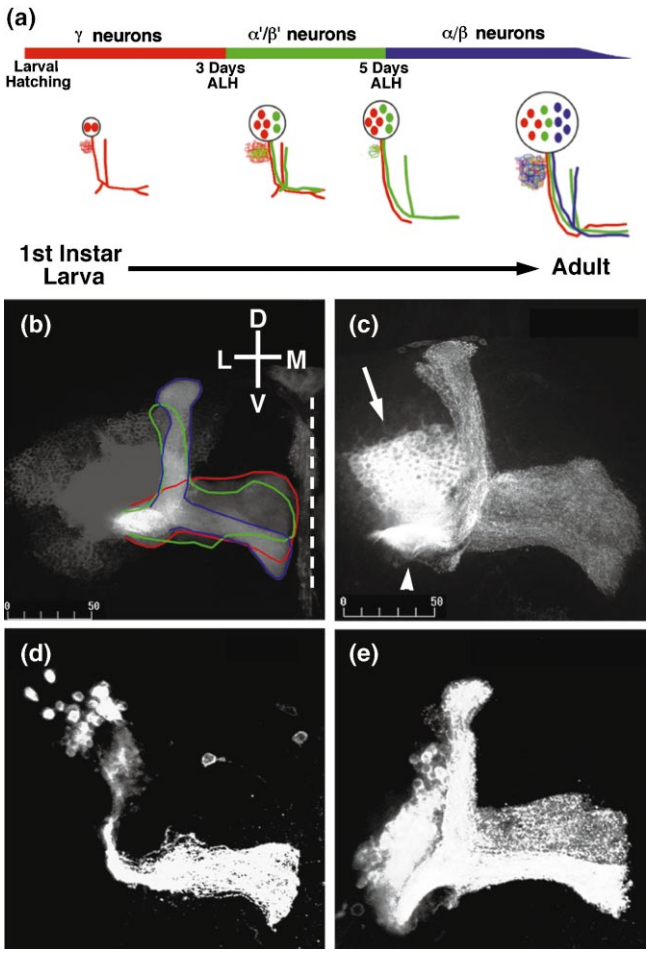
mosaic analysis in the mushroom bodies in order to identify a cellular phenotype in a well-characterized tissue [1]. The mushroom bodies are paired neuropils in the insect brain that have been shown to be important for olfactory learning and memory [9]. An adult mushroom body comprises a cell body cluster, an adjacent calyx containing the dendrites, an axon bundle called the peduncle, and five lobes that form from the axons as the peduncle defasciculates (Figure 2b). Of these five lobes, the α and α′ lobes project dorsally, and the β, β′, and γ lobes project medially [1].

The neurons composing one mushroom body are the offspring of four neuroblasts that divide throughout embryonic, larval, and pupal life [10]. These mushroom body neuroblasts divide asymmetrically to regenerate the neuroblast and create a ganglion mother cell (GMC). The GMC then divides symmetrically and gives rise to two neurons. There are three morphologically distinct types of neuron in the adult mushroom body, and the projection pattern of a given neuron depends on its birth time [1]. For the first 3 days of larval life, neurons are born that will project axons only to the γ lobe of the adult mushroom body. Neurons born from the third day of larval life until puparium formation are destined to send axons into the adult α′ and β′ lobes. Neurons in the third category are born during pupal life and project axons to the α and β lobes (Figure 2a).

We made use of the system of mosaic analysis with a repressible cell marker (MARCM, [11]) to create uniquely labeled *enok*$^{-}$ clones in the mushroom bodies. When a heat shock was used to induce FLP recombinase expression and mitotic recombination in the first 2 hr ALH, wild-type clones observed in adults contained clearly marked cell bodies, calyx, peduncle, and five lobes (Figure 2c). In contrast, *enok*$^{-}$ clones contained only what appeared to be a γ lobe and had a reduced cell number (Figure 2d); the gene therefore earns its name with reference to the mushroom with a thin stem and small cap. Phenotypes for *D23*, *enok*^{1}, and *enok*^{2} were indistinguishable (data not shown). This phenotype was rescued (Figure 2e) by a genomic transgene that starts approximately 3.9 kb upstream of the *enok* coding region and ends at the end of the neurofilament-like domain (Figure 1a). This transgene, however, failed to rescue lethality.

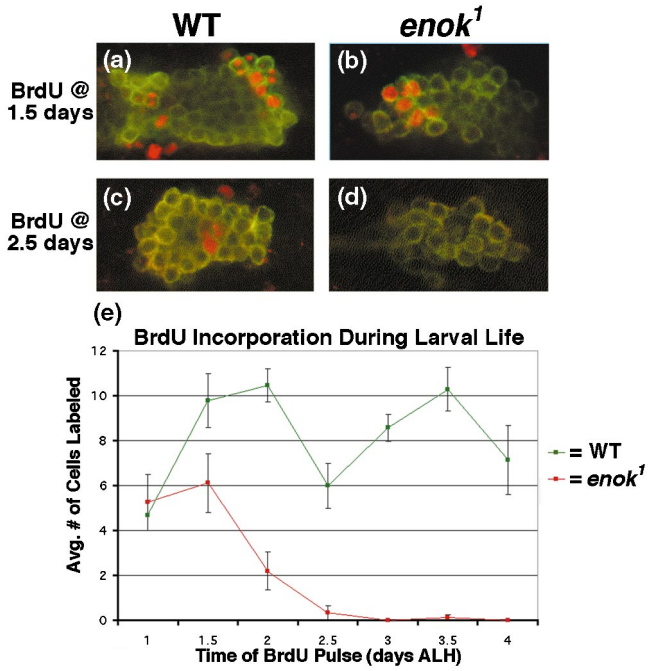
Given the way in which the mushroom bodies develop, there are three potential explanations for this phenotype. First, *enok*$^{-}$ neuroblasts might stop contributing new neurons at an abnormally early stage in development. If neurons were only born into the clone within the first three days of larval life, then all of these neurons would be expected to project to the γ lobe (Figure 2a). Second, the cell fate switch from γ-projecting neurons to α′/β′-projecting neurons that normally occurs at three days ALH might somehow be perturbed. Finally, the phenotype

Figure 2



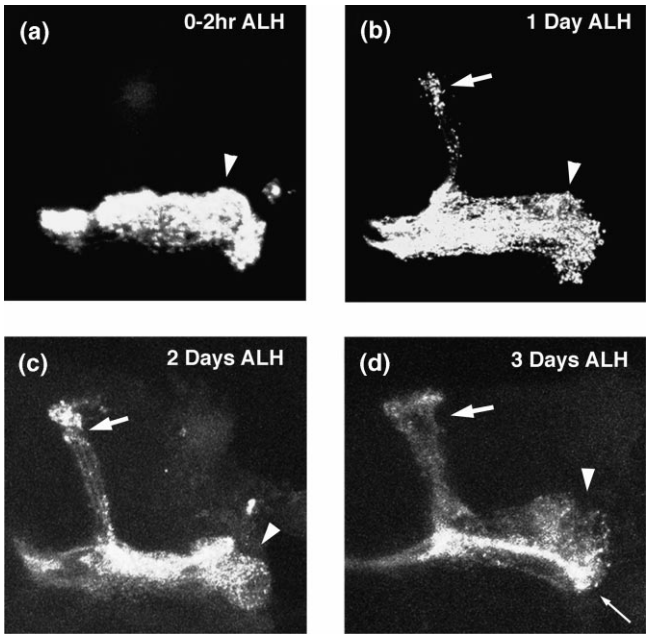
enok^{-} phenotype in the mushroom body. **(a)** provides a schematic diagram of mushroom body development, illustrating that γ neurons, α′/β′ neurons, and α/β neurons are produced sequentially by the same neuroblasts. **(b)** The structure of the entire mushroom body can be seen by expressing a mushroom body GAL4 driver (OK107) with a UAS-mCD8-GFP transgene. The γ lobe is outlined in red, the α′ and β′ lobes are outlined in green, and the α and β lobes are outlined in blue. The dorsal, ventral, medial, and lateral directions are indicated, and the dotted line shows the position of the midline. **(c)** Wild-type MARCM clones resulting from neuroblasts that undergo mitotic recombination at the beginning of larval life contain all five lobes. The cell bodies (arrow) and calyx (arrowhead) are indicated. The peduncle projects through the Z axis and is obscured by the calyx and axons. **(d)** Clones homozygous for *enok*^{1} exhibit a reduced cell number and contain only the γ lobe. **(e)** This *enok*^{1} phenotype can be fully rescued by the introduction of a genomic *enok* transgene. Two insertions of this transgene were tested, and both rescued fully (data not shown). Scale bars are in microns, and (c), (d), and (e) are shown at the same scale. Genotypes are as follows: (b) *UAS-mCD8-GFP / +*; *GAL4-OK107 / +* (c) *hs-Flp /+*; *FRT*^{G13}, *UAS-mCD8-GFP / FRT*^{G13}, *tubP-GAL80; GAL4-OK107 / +* (d) *hs-Flp /+*; *FRT*^{G13}, *UAS-mCD8-GFP, enok*^{1} */ FRT*^{G13}, *tubP-GAL80; GAL4-OK107 / +* (e) *GAL4-C155, hs-Flp / P[w*$^{+}$ *; enok rescue construct]; FRT*^{G13}, *UAS-mCD8-GFP, enok*^{1} */ FRT*^{G13}, *tubP-GAL80*. Differences in the staining intensity of certain lobes in (c) versus those in (e) result from the different GAL4 lines used. *GAL4-C155* provides pan-neural expression [1]. Frames (a) and (b) are modified from [1].

Figure 3



Timed BrdU pulses in the mushroom bodies. Following the induction of mitotic recombination at 0–2 hr ALH, pulses of BrdU were given to the larvae at specific times, and the cell body clusters of mushroom body clones were visualized. In panels (a–d), cell bodies are shown in green, and BrdU is shown in red. BrdU is incorporated into **(a)** wild-type and **(b)** *enok*^{1} clones pulsed with BrdU at 1.5 days ALH. Pulses at 2.5 days ALH incorporated BrdU into **(c)** wild-type but not **(d)** *enok*^{1} clones. Incorporation of BrdU throughout the course of larval life is summarized in **(e)**. Error bars represent SEM. Genotypes for (a) and (c) are *GAL4-C155, hs-Flp / + ; FRT*^{G13}*, UAS-mCD8-GFP / FRT*^{G13}*, tubP-GAL80*. Genotypes for (b) and (d) are *GAL4-C155, hs-Flp / + ; FRT*^{G13}*, UAS-mCD8-GFP, enok*^{1} */ FRT*^{G13}*, tubP-GAL80*.

Figure 4



enok^{1} clones generated at various times during development. As we know from previous experiments, *enok*^{1} clones induced at 0 days ALH contain only a γ lobe (arrowhead, **[a]**). When we created *enok*^{1} clones by heat shocking at 1 day ALH, we again observed a robust γ lobe (arrowhead, **[b]**), but we also began to see dorsal projections into the α′ lobe (arrow; any β′ projections in this clone are obscured by the γ lobe). When clones were induced at 2 days ALH, the α′ projections became more pronounced (arrow, **[c]**) as the γ lobe became fainter (arrowhead). Finally, *enok*^{1} clones induced at 3 days ALH had only faint traces of the γ lobe (arrowhead, **[d]**), while the α and α′ lobes (arrow) and the β and β′ lobes (small arrow) were clearly present. The time at which the clones were induced is indicated in each panel. For all panels, the genotype is *hs-Flp /+; FRT*^{G13}*, UAS-mCD8-GFP, enok*^{1} */ FRT*^{G13}*, tubP-GAL80; GAL4-*OK107/ +.

could be explained by a branching defect in which neurons that had properly defined themselves as α′/β′ or α/β would fail to send axons dorsally due to an inability to form a branch. The following experiments were intended to allow us to distinguish among these three possibilities.

enok^{-} mushroom body neuroblasts prematurely stop contributing neurons

In order to determine whether neurons were being generated normally in *enok*$^{-}$ clones, we combined the MARCM system with timed pulses of the base analog bromodeoxyuridine (BrdU). Clones were induced during the first 2 hr ALH, and BrdU pulses were given at various times during development. This allowed us to see whether neurons were still being born into the clone at the time of the pulse. Figure 3 provides representative images of cell body clusters from wild-type and *enok*^{1} mushroom body clones. While clones of both genotypes were still incorporating new neurons during a pulse of BrdU at 1.5 days ALH (Figure 3a,b), *enok*^{1} clones have stopped adding neurons by 2.5 days ALH (Figure 3d,e). In contrast, wild-type clones incorporated BrdU at 2.5 days ALH (Figure 3c) and throughout larval life (Figure 3e). This indicates that Enok is required for normal mushroom body neuroblast proliferation.

enok is not required for mushroom body cell fate switching or axon branching

While it seems likely that a defect in neuroblast proliferation is responsible for the *enok*$^{-}$ phenotype in the mushroom bodies, we do not know if defects in cell fate switching or axon branching also contribute to the phenotype. To investigate these possibilities, we induced mitotic recombination at different times and observed the types of lobes that formed in the resulting clones. We found a normal progression through γ, α′/β′, and finally α/β neurons, and we found that the transitions between cell types occurred at the normal times (Figure 4).

This normal progression through mushroom body development and, more importantly, the presence of multiple types of neurons within the same clone indicate that transitions among the three types of mushroom body neurons proceed normally in an *enok*$^{-}$ background. Furthermore, since branched α′/β′ and α/β neurons can be generated in late-induced *enok*^{1} clones (Figure 4d), it is unlikely that a defect in axon branching plays a role in the initially observed *enok* phenotype. Consistent with the results of the BrdU study (Figure 3), the cell number was reduced at all time points (data not shown), and this finding indicates that the cell proliferation defect occurs regardless of the developmental stage during which the neuroblasts are dividing.

enok^{-} clones display cell proliferation defects in some, but not all, tissues

Having established that *enok* is essential for normal cell proliferation in the mushroom bodies, we were interested in determining the generality of this requirement in various tissues. Since there are few neural tissues that have well-characterized cell lineages like that of the mushroom body, we have investigated the importance of *enok* to proliferation of nonneural cells. To this end, we used the MARCM system to create homozygous *enok*^{1} clones in the developing wing discs. We found no significant difference in the size of *enok*^{1} clones as compared to the wild type (see supplemental Figure S1) in spite of the fact that in situ hybridizations of L3 wing discs show *enok* mRNA to be present (data not shown).

The difference in requirement for *enok* seen between the mushroom bodies and wing discs may result from the tissues' different developmental programs. Any line of cells in the linearly dividing mushroom bodies would undergo many more consecutive divisions than would a line in the exponentially dividing wing discs [12, 13]. To determine whether the linear division pattern of the mushroom body progenitors is a key factor, we have induced clones in a cell type that exhibits a similarly linear division pattern, the female germ line cells [14]. Germ line clones of *enok*$^{-}$ and the wild-type control were generated with the *ovo*^{D} system [15]. This ensured that any eggs laid would be from homozygous clones in the ovaries. Throughout the life of the females, we observed a slow but steady decline in the number of eggs laid by *enok*^{1}/*ovo*^{D} females as compared to *enok*$^{+}$/*ovo*^{D} females (see Figure S2 in the Supplementary material). This slow, steady decline in egg laying may indicate a role for *enok* in germ line cell divisions, but the nature of the decline is far less dramatic than that seen from the mushroom body neuroblasts. The mortality of *enok*$^{-}$ animals makes it clear that tissues other than the mushroom bodies require *enok* function, but our experiments in the wing discs and ovaries indicate that *enok*'s requirement for cell proliferation is far from ubiquitous.

In summary, we have identified a novel *Drosophila* putative HAT that is essential for survival in general and for normal cell proliferation in particular. Importantly, *enok* appears to be responsible for some of the phenotypes originally attributed to *gek* (for details, see Figure S3 in the Supplementary material). In addition to the results described here, *enok* has been identified in a forward genetic screen for exhibiting a specific retinotopic projection defect (G. Gahmon and B. J. Dickson, personal communication). Together, these findings suggest pleiotropic and important functions of *enok* in neural development. Many HATs serve as parts of large protein complexes that regulate transcription [16]. In future studies of *enok*, it will be important to identify which proteins form a functional complex with Enok to direct its catalytic activity.

Supplementary material

Three supplementary figures, showing the wing disc clone data, the results of the oogenesis study, and the relationship between the *enok* and *gek* phenotypes, are published with the electronic version of this article at http://current-biology.supmat.com. Materials and methods can also be found on the internet.

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